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Prostate Specific Membrane Antigen (PSMA) is a transmembrane protein expressed predominantly in prostate epithelial cells. The expression of PSMA increases several fold in cancer cells, and recently it was shown that PSMA is involved in the modulation of invasiveness of prostate cancer cells.

The objective of this project was to identify a potential ligand for PSMA. For this purpose, we have employed the yeast two-hybrid system using different regions of the extracellular domain of PSMA as baits and screened a human prostate specific cDNA expression library. In this screen, we have identified three extracellular matrix components, collagen XVIII, fibulin like protein, and laminin 5 receptor like protein as potential interacting partners of PSMA.

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Introduction

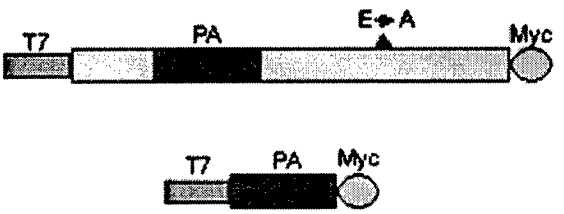
Prostate Specific Membrane Antigen (PSMA) is an integral membrane glycoprotein predominantly expressed in the prostatic epithelium (1, 2). PSMA expression is observed at elevated levels in higher grade, hormone refractory, and metastatic prostate cancers (3). Although there is a direct correlation between PSMA expression and increasing tumor aggressiveness, disease recurrence, and metastatic potential (3); the significance of increased PSMA expression in advanced prostate cancer is currently unknown. Recently, it was shown that PSMA is expressed in the neovasculature of tumor cells and not in the normal endothelial cells, suggesting a role for PSMA in the process of angiogenesis (3). The clinical usefulness of PSMA as a diagnostic marker and a potential immuno-therapeutic target for prostate cancer is well documented (4). Therefore, understanding the biological function of PSMA is very pertinent.

We have shown that the cytoplasmic tail of PSMA is required for its internalization in a clathrin-dependent manner resulting in its accumulation in a perinuclear endocytic recycling compartment like many other membrane receptors (5). We have also observed that PSMA can undergo dimerization *in vivo*. Other striking observation is the high structural identity of PSMA with transferrin receptor, a membrane receptor playing a key role in maintaining the iron homeostasis in cells. Recently, I have shown that filamin A, an actin crosslinking protein known to bind to many receptors, associates with the cytoplasmic tail of PSMA and regulates its internalization (6). The antibody raised against the extracellular domain of PSMA can enhance the rate of PSMA internalization, suggesting that the antibody can mimic a ligand (5). These observations prompted me to suggest that PSMA could function as a membrane receptor that binds a possible ligand.

Body:

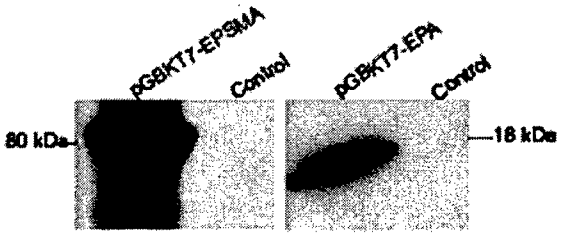
I have used the yeast two-hybrid system as a method to identify the interacting partners of PSMA. Different regions of the extracellular domain of PSMA were used as baits to screen the human cDNA expression library obtained from Clontech, Palo Alto, CA. It is known that PSMA is a peptidase belonging to the M28 peptidase family, and previous study has demonstrated the presence of a critical glutamic acid residue at the catalytic center in the 425th position. Mutation of this residue to alanine was found to abolish the enzymatic activity of PSMA (7). Based on this knowledge, I generated a point mutation within the extracellular domain (ED) of PSMA, which will render the mutant PSMA inactive in cleaving the substrate. This construct was used as one of the baits in the screening process. The highly conserved Protease Associated (PA) domain positioned close to the transmembrane domain constituted another bait (see Fig.1).

Fig. 1 Schematic representation of the baits used for the yeast-two hybrid assay. The complete extracellular domain (ED) of PSMA consisting of the protease associated (PA) domain, and the catalytic center with the glutamic acid (E) mutated to alanine (A) expressed in fusion with a myc epitope under the control of a T7 promoter constitutes one bait. The other bait consists of the PA domain alone fused with the myc epitope.



The regions of PSMA used as baits were amplified by polymerase chase reaction (PCR) using cDNA of PSMA as the target. The PCR products were cloned in frame with pGBKT7 (Clontech), a yeast two-hybrid bait vector that provides the T7 promoter and a myc epitope tag at the 3' end (Fig. 1). These recombinant vectors were subjected to *in vitro* coupled transcription and translation, and the products were analyzed by SDS-PAGE. The constructs could produce the proteins of expected molecular weight in this reaction (Fig. 2).

Fig. 2 Analysis of *in vitro* transcription translation products from yeast two-hybrid bait constructs. The bait constructs were subjected to *in vitro* coupled transcription translation reaction. The ^{35}S-methionine labeled products were resolved on a 12% SDS-PAGE, and exposed to autoradiographic film.



These constructs were transformed into a yeast strain AH 109 and used for screening the library. Several rounds of screening at various stringent conditions enabled me to narrow down some putative ligands, which include collagen XVIII, fibulin like protein, and mannose 5 receptor like protein.

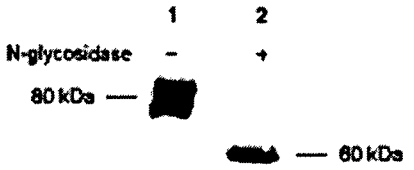
In order to determine if any of the potential binding partners identified in the yeast two-hybrid screen bind to PSMA, I have tried an *in vitro* binding assay. In this assay, I incubated the *in vitro* transcribed and translated radioactive labeled product of the clone that represents collagen XVIII with the cellular lysate containing PSMA. The binding reaction was allowed to proceed for 2 hrs at 4^0C, and the complex was pulled down using anti-PSMA antibodies. I could not see any collagen bound to PSMA as seen in Lane 2 of Fig 3.

Fig. 3 Binding of collagen XVIII with PSMA. The *in vitro* transcribed and translated ^{35}S-methionine labeled collagen XVIII was incubated with PC3 or PC3-PSMA cell lysates. Anti-PSMA antibodies were used to pull down any interacting collagen XVIII. Lane 3 (collagen XVIII) shows the input.

This can be trouble shot in two different ways. First, I will try to optimize the binding conditions. Secondly, the *in vitro* binding assay can be carried out using the catalytically inactive mutant of PSMA. This will address the question as to whether the peptidase activity of PSMA would have degraded the collagen *in vitro* because collagen is a substrate for PSMA. This problem could be addressed soon. I have already generated the catalytically inactive mutant of PSMA tagged with GFP in pEGFP-N3 (Clontech).

In parallel with yeast two-hybrid screening, I have also planned to do a biochemical-binding assay (such as far western blot analysis) for identifying the putative PSMA ligand. In this assay, the cell lysate is resolved on a SDS-PAGE and probed with the purified protein. After washing the unbound protein, the blot is subjected to regular immunoblot analysis to detect the purified protein bound to its interacting partner. The purified fully glycosylated, extracellular domain (ED) of PSMA is required for this assay. I have cloned the complete ED of PSMA in a mammalian expression vector (pSecTag, Invitrogen, Carlsbad, CA) as a C-terminal 6X histidine fusion product. The fully glycosylated ED of PSMA was purified using Nickel-NTi affinity chromatography (Fig. 4).

Fig. 4 Affinity purification of PSMA. The extracellular domain (ED) of PSMA cloned in pSecTag vector was used to transfect HEK293 cells. The secreted PSMA ED from the conditioned medium was purified using Nickel-NTi affinity chromatography. The purified protein was subjected to N-glycosidase treatment, resolved on a 10% SDS-PAGE, and stained with coomassie blue.



Key Research accomplishments:

- Identification of collagen XVIII, laminin 5 receptor like protein, and fibulin like protein as putative PSMA ligands.
- Overexpression and purification of the extracellular domain of PSMA to homogeneity by Nickel-NTi affinity chromatography.

Reportable Outcomes:

During this period, I have co-authored a review titled " Is PSMA a multifunctional protein" which is in press in **American Journal of Physiology – Cell physiology.**

I have co-authored a paper titled "N-glycosylation and microtubule integrity are involved in apical targeting of prostate specific membrane antigen: Implications for immunotherapy", which is in press in **Molecular Cancer Therapeutics.**

I have also co-authored another paper titled "Novel Role for Na,K-ATPase in Phosphatidylinositol 3-Kinase Signaling and Suppression of Cell Motility" which is published in **Molecular Biology of the Cell,** 2005 Mar;16(3):1082-94.

Conclusions:

In the present study, I have identified three potential interacting partners for PSMA *viz.*, collagen XVIII, laminin 5 receptor like protein, and fibulin like protein. All these identified proteins are related to the extracellular matrix (ECM). A recent study shows that the enzyme activity of PSMA is essential to reduce the invasiveness of prostate cancer cells in Matrigel (8). Thus an enzyme like PSMA can interact with one or more of these ECM components, and this interaction might help in the remodeling of the ECM thus modulating the motility and invasiveness of PSMA positive cells.

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